THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 46, pp. 38569–38579, November 9, 2012
Published in the U.S.A.

The Matricellular Protein CCN1/Cyr61 Is a Critical Regulator of Sonic Hedgehog in Pancreatic Carcinogenesis*[S]

Received for publication, June 6, 2012, and in revised form, September 30, 2012 Published, JBC Papers in Press, October 1, 2012, DOI 10.1074/jbc.M112.389064

Inamul Haque[‡§], Archana De[‡1], Monami Majumder[‡§1], Smita Mehta[‡], Douglas McGregor[‡¶], Sushanta K. Banerjee[‡§¶‖], Peter Van Veldhuizen[‡§], and Snigdha Banerjee[‡§2]

From the [‡]Cancer Research Unit, Kansas City Veterans Affairs Medical Center, Kansas City, Missouri 64128, and the [§]Division of Hematology and Oncology, the [‖]Department of Anatomy and Cell Biology, and the [¶]Department of Pathology, University of Kansas Medical Center, Kansas City, Kansas 66206

Background: CCN1 plays a vital role in pancreatic carcinogenesis with an unknown mechanism.
Results: CCN1 regulates Sonic-Hedgehog in pancreatic cancer cells via integrin-*Notch*-signaling pathway to promote *in vitro* motility and *in vivo* tumorigenic growth.
Conclusion: CCN1 is a critical regulator of Sonic-Hedgehog signaling in pancreatic cancer cells.
Significance: Studies suggest a mechanism whereby CCN1 regulates carcinogenic events in the pancreas.

CCN1 is a matricellular protein and a member of the CCN family of growth factors. CCN1 is associated with the development of various cancers including pancreatic ductal adenocarcinoma (PDAC). Our recent studies found that CCN1 plays a critical role in pancreatic carcinogenesis through the induction of EMT and stemness. CCN1 mRNA and protein were detected in the early precursor lesions, and their expression intensified with disease progression. However, biochemical activity and the molecular targets of CCN1 in pancreatic cancer cells are unknown. Here we show that CCN1 regulates the Sonic Hedgehog (SHh) signaling pathway, which is associated with the PDAC progression and poor prognosis. SHh regulation by CCN1 in pancreatic cancer cells is mediated through the active *Notch-1*. Notably, active *Notch-1*is recruited by CCN1 in these cells via the inhibition of proteasomal degradation results in stabilization of the receptor. We find that CCN1-induced activation of SHh signaling might be necessary for CCN1-dependent *in vitro* pancreatic cancer cell migration and tumorigenicity of the side population of pancreatic cancer cells (cancer stem cells) in a xenograft in nude mice. Moreover, the functional role of CCN1 could be mediated through the interaction with the $\alpha v\beta 3$ integrin receptor. These extensive studies propose that targeting CCN1 can provide a new treatment option for patients with pancreatic cancer since blocking CCN1 simultaneously blocks two critical pathways (*i.e.* SHh and *Notch1*) associated with the development of the disease as well as drug resistance.

Pancreatic ductal adenocarcinoma (PDAC),[3] with a prevalence of 2–3% of new cancer cases annually in the United States, is the fifth leading cause of cancer deaths in the United States and globally (1–4). Prognosis of PDAC is extremely dismal. Due to the impalpable nature of the disease, PDAC is hard to diagnose at an early stage and typically presents with metastasis at the time of diagnosis. Currently, there are no effective therapies for PDAC, and it exhibits a profound resistance to current chemotherapies (5). Therefore, new insights into the etiology of PDAC progression along with its precise mechanisms of drug resistance need to be discovered.

PDAC develops from pancreatic intraepithelial neoplastic (PanIN) precursor lesions through multiple histologic, genetic, and epigenetic changes (6, 7). Multiple studies have found that sonic hedgehog (SHh), a lipid-modified, secreted signaling protein, plays a critical role in PDAC's development from PanIN lesions to the invasive growth of the disease (8–12). Aberrant expression of SHh in pancreatic ductal epithelial cells, which is normally absent in the developing and mature pancreas (13), binds to the receptor patched (PTCH) to prevent an inhibitory impact of PTCH on the smoothened (Smo) receptor. The released and activated Smo then promotes translocation of GLI family of transcription factors from cytoplasm to the nucleus to induce the expression of SHh-targeted genes linked with carcinogenic events in the ducts of the pancreas (10, 11, 14, 15). The GLI-independent pathway may also be involved in SHh mediated pancreatic carcinogenesis (14). Despite the discrepancy in the literature, the previous studies have demonstrated that SHh is responsible for PDAC cell proliferation, epithelial-mesenchymal transition (EMT), maintenance of cancer stemness, migration, invasion, and metastatic growth in distant organs (5, 10, 11, 15–19). Moreover, SHh also plays a critical role in promoting desmoplasia and drug resistance in animal models (11, 20, 21). Notwithstanding knowledge of all these pathobiological impacts of SHh-signaling in PDAC, the mechanism(s) whereby SHh is activated in pancreatic cancer cells remains elusive. Insight into this regulation could provide a new rationale for improved therapy against this disease.

CCN1, which is also known as Cyr61 (cysteine-rich 61), is a member of the CCN family of matricellular proteins which consists of CTGF, NOV, WISP-1, WISP-2, and WISP-3 (22–26).

* This work was supported by a Merit review grant from the Dept. of Veterans Affairs (to S. B. and S. K. B.).
[S] This article contains supplemental Table S1 and Figs. S1 and S2.
[1] Both authors contributed equally to this work.
[2] To whom correspondence should be addressed: Cancer Research Unit, Research Division, V.A. Medical Center, 4801 Linwood Blvd, Kansas City, MO 64128. Tel.: 816-861-4700; E-mail: sbanerjee@kumc.edu/sbanerjee2@kumc.edu/publication@vamccancerresearchunit.org.
[3] The abbreviations used are: PDAC, pancreatic ductal adenocarcinoma; SHh, Sonic Hedgehog; Smo, smoothened; SP, side population; NSP, non-side population; ICD, intracellular domain.

CCN1 is a secretory, multifunctional protein, growth factor inducible, and an immediate early response gene (27). CCN1 is either localized intracellularly or associated with the cell surface and extracellular matrix, and it is involved in the adhesion, proliferation, migration, differentiation and angiogenesis during normal and patho-physiological processes (23, 24). The histopathological and immunohistochemical studies indicate that, except in lung cancers (28) and leiomyomas (29), CCN1 expression is markedly increased in different human cancers including PDAC (7, 30, 31). Our recent studies show that CCN1, when overexpressed in PDAC and its precursor lesions, promotes proliferation, EMT, and migration of pancreatic cancer cells and, possibly, regulates stemness of these cells through the regulation stemness regulatory genes and microRNAs (7). However, it remains unknown how the CCN1 system becomes rewired at the molecular and cellular levels to promote PDAC growth.

Given that SHh and CCN1 signaling are associated with the genesis of human PDAC, one could speculate that these signaling molecules walk hand-over-hand or their regulation is mutually dependent for the development of PDAC. These studies support the hypothesis and show that CCN1 is an upstream regulator of SHh in pancreatic carcinogenesis. Our data also provide evidence that integrin $\alpha v\beta 3$-*Notch1* signaling is critical in CCN1 induced SHh expression in pancreatic cancer cells. Collectively, these studies illustrate that CCN1 could be an ideal target in pancreatic cancer cells to prevent the action of two critical signaling cascades.

MATERIALS AND METHODS

Cell Lines and Cell Culture—Human pancreatic cancer cell lines *i.e.* BxPC-3, Capan-1, AsPC-1, Hs766T, Panc-1 and MIA-PaCa-2 were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma), supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin (Sigma) in a 37 °C incubator in the presence of 5% CO_2. CCN1-silenced Panc-1 and MIA-PaCa-2 cell lines were prepared and maintained by our laboratory (7). Ampho-pak 293 packaging cell line was purchased from Clontech and was maintained in high glucose DMEM containing 10% FBS. Cells were used for the experiment between four and six passages.

Reagents and Antibodies—Human polyclonal anti-rabbit CCN1 antibody, rabbit polyclonal anti-human *Notch-1*, mouse monoclonal anti-human CD24, human polyclonal anti-goat Jagged-1, polyclonal goat anti-rabbit IgG-HRP, human polyclonal anti-rabbit *Notch-1* and monoclonal goat anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-mouse GAPDH antibody was purchased from Applied Biosystems (Foster City, CA). Human monoclonal anti-rabbit SHh antibody, Mouse monoclonal Collagen 1, and human polyclonal anti-rabbit Ptch antibody were obtained from Abcam (Cambridge, MA). Human rabbit polyclonal Gli1 was purchased from Cell Signaling (Boston, MA). Cyclin D1 and Bcl-2 antibodies were purchased from BD Biosciences (San Jose, CA) and Calbiochem respectively. pSilencer™ 5.1-U6 retroviral vector and siPORT™ XP-1 transfection agent were obtained from Applied Biosystems (Foster City, CA). All other chemicals were obtained either from Sigma or Fisher Scientific (Houston, TX). Cyr61 recombinant protein was purchased from Fisher Scientific (St. Louis, MO). *Notch-1* inhibitor DAPT [*N*-(*N*-(3, 5-difluorophenacetyl)-L-alanyl)-*S*-phenylglycine *t*-butyl ester] was purchased from Sigma. Matrigel was purchased from BD Biosciences (San Jose, CA). Cyclopamine was obtained from Sigma.

Mouse Xenograft Experiments—The animal studies were conducted according to the approved Guidelines of the Animals Care and Use Committee of Kansas City VA Medical Center. For subcutaneous nude mice xenograft studies, side population (SP), and non-side population (NSP) of Panc-1 cells were isolated by a BD FACS Aria SORP flow cytometer (BD Biosciences) using ~405 nm excitation and 440 nm emission as described previously by Haque *et al.* (7). Sorted cells (*i.e.* SP and Non-SP) were briefly cultured in DMEM with 10% FCS in 5% CO_2 at 37 °C, and then cells (5×10^4 cells suspended in Matrigel to a final volume of 100 μl) were injected *s.c.* into the right rear flank of 6–8-week-old male athymic nude mice (6 mice per group) and tumor growth was monitored starting after the 2nd day of injection. This was continued for up to 45 days or more using our previous methods (32, 33). Male athymic nude mice (nu/nu genotype) were obtained from Charles Rivers (Wilmington, MA) and acclimated to our facility for 1 week before starting the experiments.

Retroviral Production and Transduction of Cells—CCN1-knock-out or scrambled pancreatic cancer cell lines (MIA-PaCa-2^{CCN1KO} and Panc-1^{CCN1KO}) were generated using pSilencer™ 5.1-U6 Retro-viral system (Ambion, Grand Island, NY) as per the protocol described earlier (7). Briefly, cloned human CCN1-shRNA/scrambled vector were transfected into an Amphopak™293 packaging cell line using siPORT™ XP-1 transfection agent. After transfection, the culture medium was changed and cells were incubated 48 more hours prior to collection of viral particles. Approximately, 60% of cells were infected with CCN1-shRNA containing viral supernatant or scrambled control and incubated for 72 h. Stable transfected clones were selected by puromycin treatment until the uninfected cells died. Stable cells were then cultured in regular DMEM with 10% FBS and harvested for Western or Northern blot analysis to check the transfection efficiency.

Western Blot Analysis—Cell lysates prepared from pancreatic cancer cell lines and tumor xenografts containing 30–50 μg proteins were analyzed by Western blot using the appropriate antibodies according to the method described previously (34). Signals were detected with Super Signal Ultra Chemiluminescent substrate (Pierce) using ID Image Analysis software Version 3.6 (Eastman Kodak Company, Rochester, NY).

Immunohistochemistry—Immunohistochemistry was performed on 4% formalin-fixed, paraffin-embedded tissue sections according to our previous method (7, 35). Briefly, tissue sections were de-paraffinized in Xylene, rehydrated in different grades of alcohol, washed with PBS, and blocked with tissue blocker (Zymed Laboratories Inc.) for 10 min, and then immunostained by specific antibodies overnight in a moist chamber. The immunoreactivity was detected by conjugated streptavidin, and the sections were counterstained with hematoxylin.

The sections were imaged with a Leica photomicroscope. All samples were used according to VA Medical Center and University guidelines after receiving Institutional Review Board approval.

Immunofluorescence—The immunofluorescence assay was carried out as described earlier (32, 36). Cells were plated in chambered slides, fixed in methanol and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. After using a blocking solution, the cells were incubated with monoclonal mouse anti-SHh overnight at 4 °C and incubated with a goat anti-mouse FITC-conjugated Alexa Fluor 488 secondary antibody. Cells were washed with 1× PBS and mounted in PBS glycerin. Immunofluorescent-stained cells were visualized using a Nikon Eclipse TE-300 microscope, and images were analyzed by software. Cells incubated without the primary antibody were treated as negative controls.

Scratch Wound Healing Assay—The motility behavior of the cells of different experimental conditions was examined by the scratch wound healing assay. Briefly, different cells were seeded in the chamber slides and allowed to reach 70–80% confluence as a monolayer and then scratched with a pipette tip diagonally. After scratching, chambers were gently washed with fresh media to remove detached cells. Migration into this area was documented and measured after 24 h. Each analysis was repeated three times.

Proteasome Activity Assay—To determine the chymotrypsin-like proteasome activity, 20 S proteasome activity assay kit (EMD Millipore, Billerica, MA) was used according to the manufacturer's instructions. Briefly, harvested cells were incubated on ice for 30 min in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 0.1% SDS, 150 mM NaCl, 1% Nonidet P-40, and a protease inhibitor mixture including 1 μg/ml aprotinin, 1 μg of leupeptin, and 1.0 mM PMSF. Cellular debris was removed by centrifugation (18,000 × *g*, 1 h, 4 °C), and the supernatant was collected for the assay. For each reaction, 50 μg of sample proteins were used in assay buffer (50 mM Tris-HCl (pH 7.5), 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$) where the chymotrypsin fluorogenic substrate *N*-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC) was added to each well at a concentration of 50 μM, and the plates were incubated for 1 h in the dark at 37 °C. The fluorescence of AMC due to proteasome-mediated cleavage of the fluorogenic substrate was measured by excitation at 360 nm and emission at 460 nm in a SynergyTM HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT).

Statistical Analysis—All experiments were performed in triplicate for each observation. Each of the data represent the mean ± S.E. from the three separate experiments. Statistical analysis was performed between the two groups of data by an unpaired Student's *t* test GraphPad Prism 4 software). Values were considered statistically significant at $p < 0.05$.

RESULTS

Differential Expression of SHh and CCN1 in Different Pancreatic Cell Lines and PDAC Samples—To determine the status of SHh and CCN1 protein in different pancreatic cancer cell lines, we evaluated the level of SHh and CCN1 in different pancreatic cell lines (*i.e.* BxPC-3, Capan-1 (less aggressive), AsPC-1, Hs766T, Panc-1, and MIA-Paca-2 (highly aggressive) (7, 37)) by Western blot analysis using specific antibodies. We found that SHh and CCN1 are highly expressed in all cell lines except BxPC-3 cells where expressions of SHh and Cyr61/CCN1 were minimal or undetected (Fig. 1*A*). The highest levels of expressions of both proteins were detected in the Panc-1 cell line.

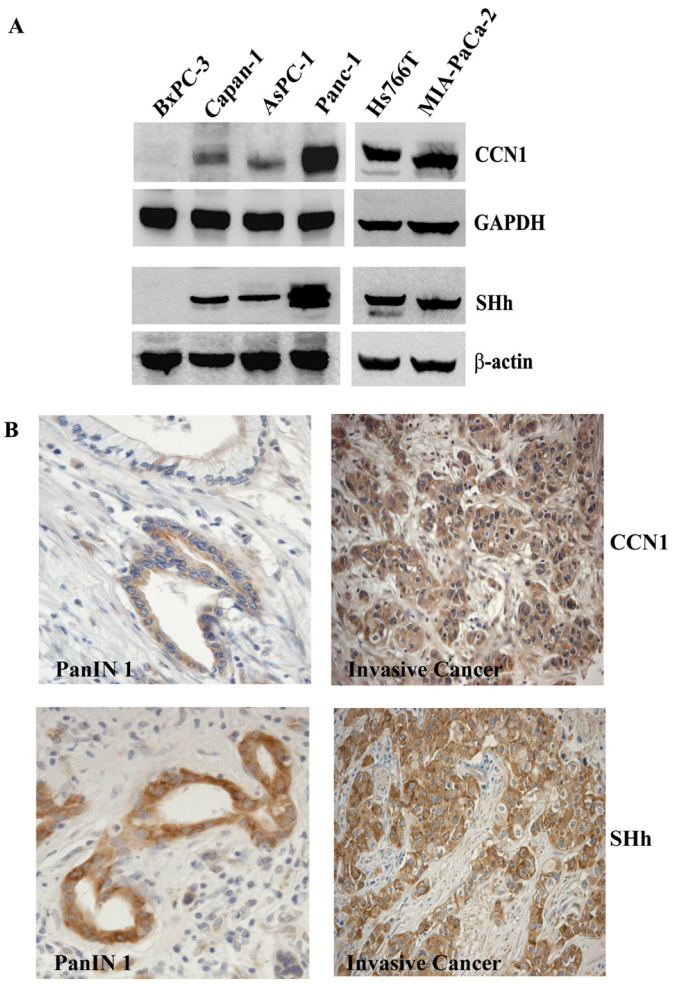

FIGURE 1. **Differential expression of CCN1 and SHh proteins in pancreatic cancer cell lines and tissue samples.** *A*, Western blots were performed with indicated antibodies in different pancreatic cell lines. CCN1 and SHh were normalized against the level of GAPDH and β-actin, respectively. The results were confirmed by three independent experiments. *B*, immunohistochemical staining showing the distribution pattern of CCN1 and SHh in PanIN1 and invasive human pancreatic cancer tissue array samples.

Next, we determined the expression profiles of CCN1 and SHh immunohistochemically in PDAC tissue arrays. Consistent with previous work (7, 14), both CCN1 and SHh were confined to the cytoplasm and their expressions were first detected in histologically defined precursor lesions (PanINs; PanIN-1A-PanIN-3) (data not shown), and the expression was markedly increased in the advanced stages of the disease (Fig. 1*B*).

CCN1 Regulates SHh Signaling in Pancreatic Cancer Cells—Recent studies have shown that SHh is an upstream regulator of CCN1 in breast cancer cells (39). Therefore, we sought to corroborate the reduced production of CCN1 protein in functionally deficient SHh pancreatic cancer cells. To do so, Panc-1 cells were exposed to a SHh receptor SMO inhibitor (39), Cyclopamine, with different doses (1–5 μg/ml) for 48 h,

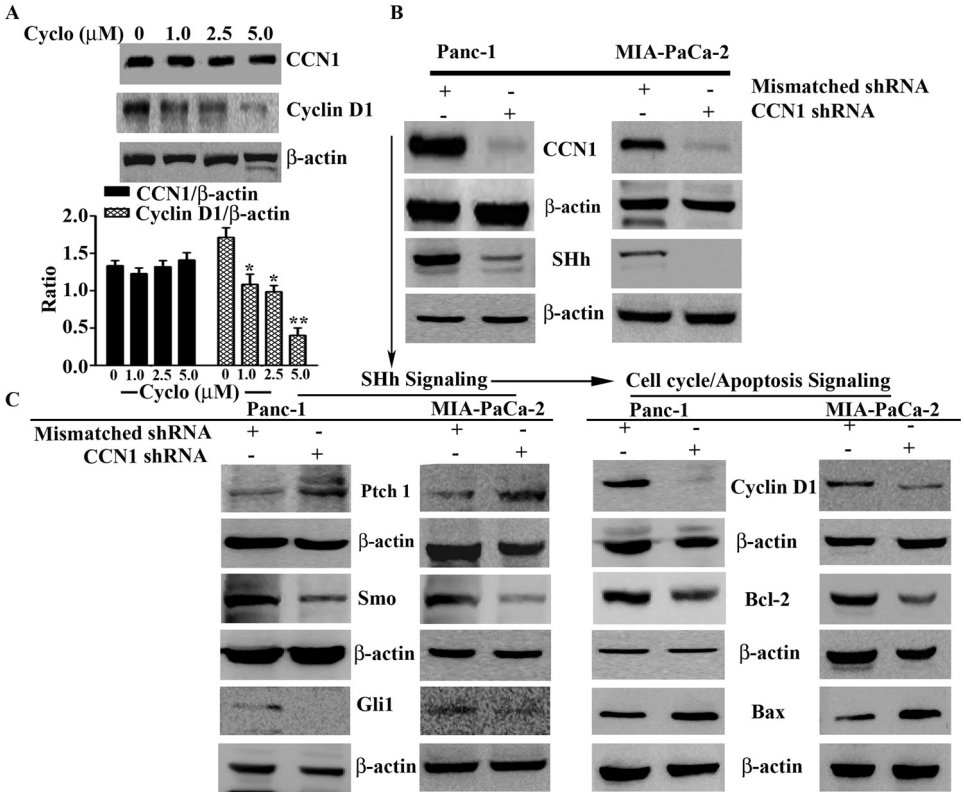

FIGURE 2. **CCN1 silencing modulates the SHh signaling pathway in aggressive pancreatic cancer cell lines.** *A*, representative Western blots showing the status of CCN1 and cyclin D1 in Cyclopamine (1, 2.5 and 5.0 μM)-treated and untreated Panc-1 cell lysates. Error bars indicate ± S.D. of three independent experiments. *, $p < 0.024$ *versus* untreated controls, **, $p < 0.001$ *versus* untreated controls. *B*, representative Western blots illustrating the effect of shRNA-mediated CCN1 silencing on SHh expression in Panc-1 and MIA-PaCa-2 cells. Nontargeting shRNA (mismatched)-transfected cells were used as CCN1-positive cell lines. *C*, representative Western blots (*left side*) represent the expression profiles of SHh signaling cascades in mismatched and CCN1-silenced Panc-1 and MIA-PaCa-2 cell lines. Representative Western blots (*right side*) show the expression of cell cycle regulatory proteins in mismatched and CCN1-silenced Panc-1 and MIA-PaCa-2 cell lines. Note, the statistical variations were determined and presented as bar graphs in supplemental Fig. S1.

and CCN1 and the downstream target of the SHh molecule cyclin D1 expression was measured using Western blotting. Unexpectedly, in contrast to the previous studies (39), we found that Cyclopamine was unable to block CCN1 expression in Panc-1 cells; however, it blocked the expression of Cyclin D1 in these cells (Fig. 2*A*). Furthermore, Panc-1 cells treated with SHh neutralizing antibody showed no effect on CCN1 expression in these cells (data not shown), demonstrating that the SHh signaling is not an upstream regulator of CCN1, at least not in Panc-1 cells. Next, we examined the status of SHh in CCN1-deficient Panc-1 and MIA-PaCa-2 cells. Thus, we generated stable CCN1 knock-out Panc-1 and MIA-PaCa-2 cell lines (designated as Panc-1^{CCN1KO} and MIA-PaCa-2^{CCN1KO}) by silencing CCN1 with CCN1-shRNA in these cells retrovirally (7) and examined the status of CCN1 and SHh using Western blot analysis. We found significantly reduced levels of CCN1 and SHh in the total cell lysates of Panc-1^{CCN1KO} and MIA-PaCa-2^{CCN1KO} cells as compared with mismatched-shRNA-transfected cells (Fig. 2*B*).

To exert biological and pathobiological functions, SHh binds with its receptor 12-span-transmembrane protein Patched (Ptch1) in a paracrine manner and relieves another 7-span-transmembrane protein receptor, Smoothened (Smo), which in turn induces the signal transduction pathway by activating the nuclear translocation of transcription factor Gli1 protein (11, 19). Therefore, in this study, we next tested whether CCN1 deficiency alters the basal level of Ptch1, Smo, and/or Gli1 in pancreatic cancer cells as a consequence of inhibiting SHh. We found that Ptch1 is up-regulated while levels of Smo and Gli1 were reduced markedly in the cell lysates of CCN1-deficient Panc-1 and Mia-PaCa-2 cells (Fig. 2*C*, *left panel* and supplemental Fig. S1).

The SHh-Ptch1-Smo-Gli1 signaling pathway promotes pancreatic ductal epithelial cell proliferation through transcription regulation of cyclin D1 and other cell cycle regulatory genes. In addition, the SHh-Ptch1-Smo-Gli1 signaling pathway protects pancreatic ductal epithelial cells from apoptotic cell death through the activation of molecules associated with Bcl-2 family for carcinogenic development (10, 19). Given that cyclin D1 and Bcl-2 are important SHh-target genes, we sought to determine whether CCN1 silencing reduces the expression of cyclin D1 and Bcl-2 in Panc-1 and MIA-PaCa-2 pancreatic cancer cells. To do so, the levels of cyclin D1, Bcl-2, and Bax were determined in Panc-1^{CCN1KO} and MIA-PaCa-2^{CCN1KO} cells using immune-Western blotting. We found both cyclin D1 and Bcl-2 levels were markedly reduced in CCN1-deficient cells (Fig. 2*C*, *right panel* and supplemental Fig. S1), while Bax expression is elevated in CCN1-silenced cells as compared with mismatched cell lines.

The Panc-1 side population (SP), which is also considered to be cancer initiating cells/cancer stem cells, produced an *s.c.* tumor with overexpressed CCN1 in nude mice within a brief

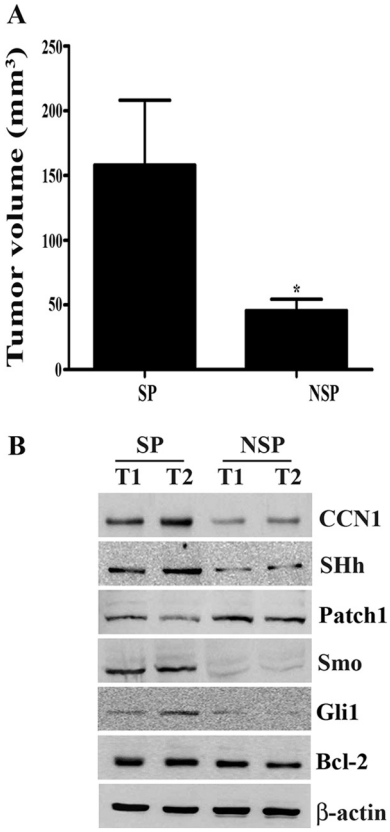

FIGURE 3. **SP of Panc-1 cell line exhibits higher tumor growth potential along with the overexpression of SHh signaling cascades than the NSP of Panc-1 cell line *in vivo*.** *A*, SP and NSP cells were separated and propagated according to our previous method, which has been described in the "Materials and Methods." Semiconfluent cells were injected *s.c.* into the right rear flank of athymic nude mice and tumor growth was monitored The bar graph represents the size of the tumors after 45 days of injection of SP and NSP Panc-1 cells ($n = 6$ mice/exp). Error bars indicate ± S.D. *, $p < 0.001$ *versus* SP. *B*, photomicrographs represent the status of CCN1, SHh, and SHh signaling cascades in SP- and NSP-tumor xenografts. T1 and T2 represent tumor no. 1 and 2, respectively.

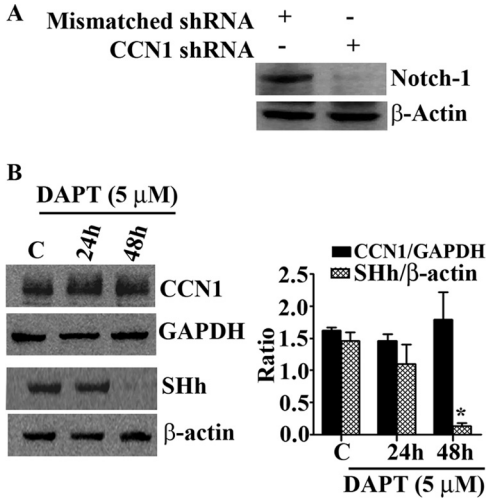

FIGURE 4. **CCN1 knockdown suppresses active *Notch-1* expression while Notch/γ-secretase inhibitor (DAPT) blocks SHh expression without affecting CCN1 expression in Panc-1 cells.** *A*, Panc-1 cells were transfected with nontargeting shRNA (mismatched) or shRNA targeting CCN1. After 48 h, cells were harvested, and cell lysates were analyzed for *Notch-1*(ICD) and β-actin protein expression by Western blotting using specific antibodies. *B*, Panc-1 cells were treated with DAPT (5 μM) for 24 and 48 h or left untreated. Cells were harvested at indicated times for Western blot with CCN1, SHh, GAPDH, and β-actin antibodies. The results of CCN1 and SHh are normalized by the intensities of GAPDH and β-actin respectively. Error bars indicate ± S.D. of three independent experiments. *, $p < 0.001$ *versus* controls.

time period as compared with the non-side population (NSP). Targeting CCN1 by shRNA most effectively reduced this feature of SP cells (7). In this study, we investigated whether SHh signaling pathways are active in SP- and NSP-xenografts. To do so, SP- and NSP-xenografts were established by injecting cells subcutaneously into the flanks of athymic nude mice. When SP-tumors reached ~200 mm^3, they were compared for volume with NSP-tumors. Significantly, SP-tumors exhibited 3-fold more growth than NSP-tumors (Fig. 3*A*). On day 45, the tumors were excised, and total proteins were extracted for Western blot analysis using CCN1, SHh, PTCH-1, Gli-1, and Smo specific antibodies. Like the corresponding *in vitro* studies, levels of SHh and *Notch-1* signaling proteins were elevated significantly in CCN1-overexpressed SP-tumors as compared with NSP-tumors where CCN1 is minimally expressed (Fig. 3*B*).

CCN1 Modulates SHh Expression through Notch-1 in Pancreatic Cancer Cells—The objective of the present work was to dissect the mechanism by which CCN1 regulates SHh expression in pancreatic cancer cells. Previously, we established that CCN1 activates *Notch-1* (the released form of an intracellular domain (ICD) of *Notch-1* into the cytoplasm) in pancreatic cancer cells and their CCN1-positive SP (7). Two independent studies have shown that *Notch-1* activates SHh to reinforce the cell-fate switch in *Xenopus* (40), and that SHh regulates *Notch-1*-targeted genes in vascular smooth muscle cells (41). These two studies, although not related to pancreatic cancer, caused us to speculate that *Notch-1* may be a requisite downstream mediator of CCN1-induced overexpression of SHh in pancreatic cancer cells. To test the hypothesis, first we determined the status of active *Notch-1*(ICD) in CCN1-deficient Panc-1^{CCN1KO} cells by Western blot analysis using an ICD-specific antibody. Consistent with previous studies (7), our present finding demonstrating that CCN1 enhances the expression of active *Notch-1*(ICD) in Panc-1 cells since shRNA-mediated silencing of CCN1 resulted in a near complete loss of active *Notch-1*(ICD) protein expression in Panc-1 cells (Fig. 4*A*), without affecting the transcription of *Notch-1* as demonstrated by qPCR analysis (data not shown). Next, we tested if active *Notch-1* is requisite for CCN1-mediated activation of SHh in pancreatic cancer cells. To do so, Panc-1 cells were treated with a pharmacological inhibitor of ICD-releasing proteolytic enzyme γ-secretase (DAPT, 5 μM) (42) or vehicle for different times (24, 48, and 72 h), and the levels of CCN1 and SHh were determined in the supernatants of tissue extracts using Western blotting. Studies showed that the expression of SHh was markedly diminished by DAPT at 48 h of treatment (Fig. 4*B*). We found, however, that treatment of DAPT has no effect on CCN1 expression in Panc-1 cells (Fig. 4*B*). Therefore, we can conclude that Notch-1 is an intermediate molecule of CCN1 and SHh and may play a vital role in CCN1-mediated activation of SHh-signaling.

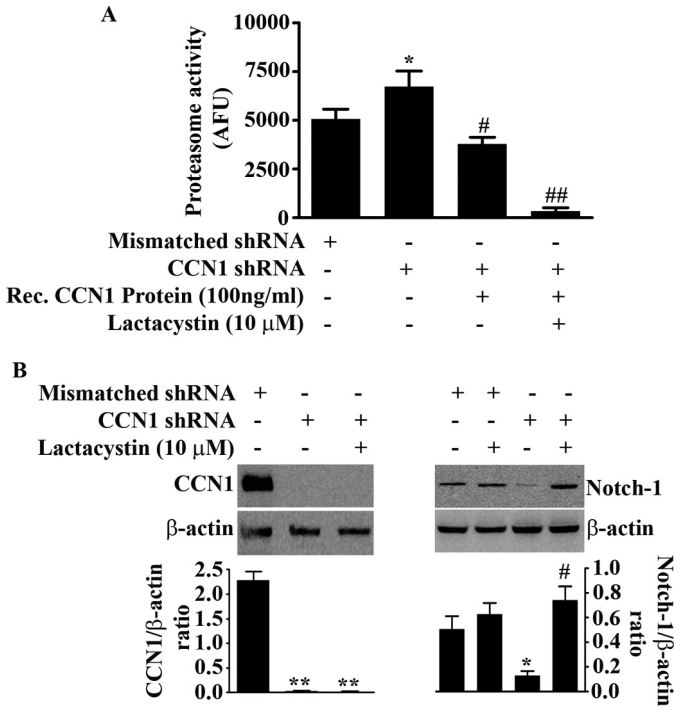

FIGURE 5. **CCN1 inhibits proteasomal activity and Notch1 degradation.** *A*, proteasomal activity (chymotrypsin-like peptidase activity) in Panc1 cells under different treatment conditions was determined by the fluorogenic substrate Suc-LLVY-AMC using 20 S proteasome activity assay kit. Proteasomal activity is expressed in arbitrary fluorescence units (AFU). Error bars indicate ± S.D. of three independent experiments. *, $p < 0.005$ *versus* mismatched shRNA; #, $p < 0.005$ *versus* CCN1 shRNA; ##, $p < 0.001$ *versus* CCN1 shRNA. *B*, representative Western blots show expression CCN1 (*left panel*) and *Notch-1* (ICD) (*right panel*) in Panc-1 cells under different treatment conditions, histograms in the *lower left panel* and *lower right panel* show the CCN1 to β-actin ratio and Notch-1 to β-actin ratio, respectively. Error bars indicate ± S.D. of three independent experiments. *, $p < 0.005$ *versus* mismatched shRNA; **, $p < 0.001$ *versus* mismatched shRNA; #, $p < 0.001$ *versus* CCN1 shRNA.

Regulation of Notch-1(ICD) by CCN1 in Pancreatic Cancer Cells—To dissect the functional role of CCN1 in regulation of *Notch-1*, we asked whether CCN1 enhances the stability of active *Notch-1*(ICD) by blocking the proteasomal degradation via the ubiquitin-proteasome system that have been implicated in the regulation of the half-life of *Notch-1* (43–45). To this aim, first, we determined the effect of CCN1 on 20 S proteasome, a catalytic core of proteasome complex. To do so, Panc-1^{CCN1KO} cells were treated with recombinant CCN1 protein (100 ng/ml) for 48 h or lactacystin (10 μM) for 24 h, and proteasome activity was determined using 20 S proteasome activity assay. We found that in the absence of CCN1, the proteasome activity was significantly increased in Panc-1 cells. In contrast, addition of the recombinant CCN1 protein in the Panc-1^{CCN1KO} cell culture reduces the proteasome activity in these cells (Fig. 5*A*). An additive effect was detected when cells were exposed to CCN1 recombinant protein along with a proteasome inhibitor Lactacystin (Fig. 5*A*). Based on this result, we proposed that CCN1 may act as an endogenous proteasome inhibitor in pancreatic cancer cells.

Given that the CCN1 protein acts as a proteasome inhibitor, next, we used above experimental strategy to investigate if Lactacystin is able to rescue *Notch-1* (ICD) from proteasomal degradation in Panc-1^{CCN1KO} cells. As shown in Fig. 5*B*, *Notch-1*(ICD) protein expression in Panc-1^{CCN1KO} cells was expectedly reduced (*right panel*, *lane 3*) as compared with the mismatched shRNA stable transfected panc-1 cells (*right panel*, *lane 1*). However, the expression of *Notch-1*(ICD) in the Panc-1^{CCN1KO} cells can be recovered by treating the cells with Lactacystin for 24 h. Lactacystin-treated cells exhibit slightly more expression of *Notch-1* (*right panel*, *lane 4*) as compared with the mismatched shRNA stable transfected panc-1 cells (*right panel*, *lane 1*). Lactacystin has no impact on CCN1 expression (*left panel*, *lane 3*). Based on the results, the studies indicate that CCN1 enhances the stability of *Notch-1*(ICD) by preventing the proteasomal degradation events in the pancreatic cancer cells.

CCN1-induced Notch-1 Activation and SHh Expression Are Mediated through Integrin αvβ3 Receptor in Pancreatic Cancer Cells—Multiple activities of CCN1 are mediated through heterodimeric cell surface integrin receptors (24). Interestingly, αv subunit of integrin is prime mediator of cellular activities of CCN1 in different cancer cells (24, 25). Given αv, we sought to determine if this unit is involved in CCN1-induced activation of *Notch-1* and SHh expression in pancreatic cancer cells. To do so, first we determined the status of different subunits of integrin in different pancreatic cell lines by Western blotting using specific antibodies. We found that all tested integrins, except α4 and α6, were expressed in Panc-1 cells, while these integrins were differentially expressed in ASPC-1 and Mia-PaCa-2 cell lines (Fig. 6*A* and supplemental Table S1). Next, to investigate the possible role of integrin αv in CCN1-mediated regulation of *Notch-1* and/or SHh expression, functional blocking monoclonal antibody (mAb) against αv was used to study if CCN1 interacts with αv to activate *Notch-1* followed by SHh expression in Panc-1. As expected, αv mAb markedly blocked CCN1-induced activation of *Notch-1* and SHh expression in Panc-1 cells (Fig. 6, *B--D*). Furthermore, an antibody of β3 (B3A) but not β1 subunits of integrins significantly abolished the activity of CCN1 on *Notch-1* activation and SHh expression in Panc-1 cells (Fig. 6, *E* and *F*). Together, these studies suggest that the integrin αvβ3 heterodimer may play critical role in CCN1-induced activation of SHh signaling.

CCN1-induced Pancreatic Cancer Cell Motility Is Mediated through SHh—Previously, we demonstrated that CCN1 is one of the prime regulators of *in vitro* migration and invasion of pancreatic cancer cells (7). SHh-signaling also promotes motility and invasiveness of gastric and pancreatic cancer cells (46, 47). Therefore, we speculate that CCN1-induced pancreatic cancer cell motility is mediated through SHh-signaling. To test the hypothesis, we used Panc-1^{CCN1KO} and MIA-PaCa-2^{CCN1KO} cells as well as Panc-1 side population (SP, CCN1-positive cells) and Panc-1 non-side population (NSP, CCN1-negative cells) cells. First, using a scratch wound assay, we determined the motile behavior of above mentioned cell lines. We observed an enhanced motility in CCN1 positive cells in comparison to CCN1-negative cells which exhibited a markedly reduced rate of wound closure after 24 h of culture in an identical culture environment (Fig. 7, *A–C*).

To test if CCN1 recombinant protein could promote motility of CCN1-deficient cells through SHh signaling, we assayed the

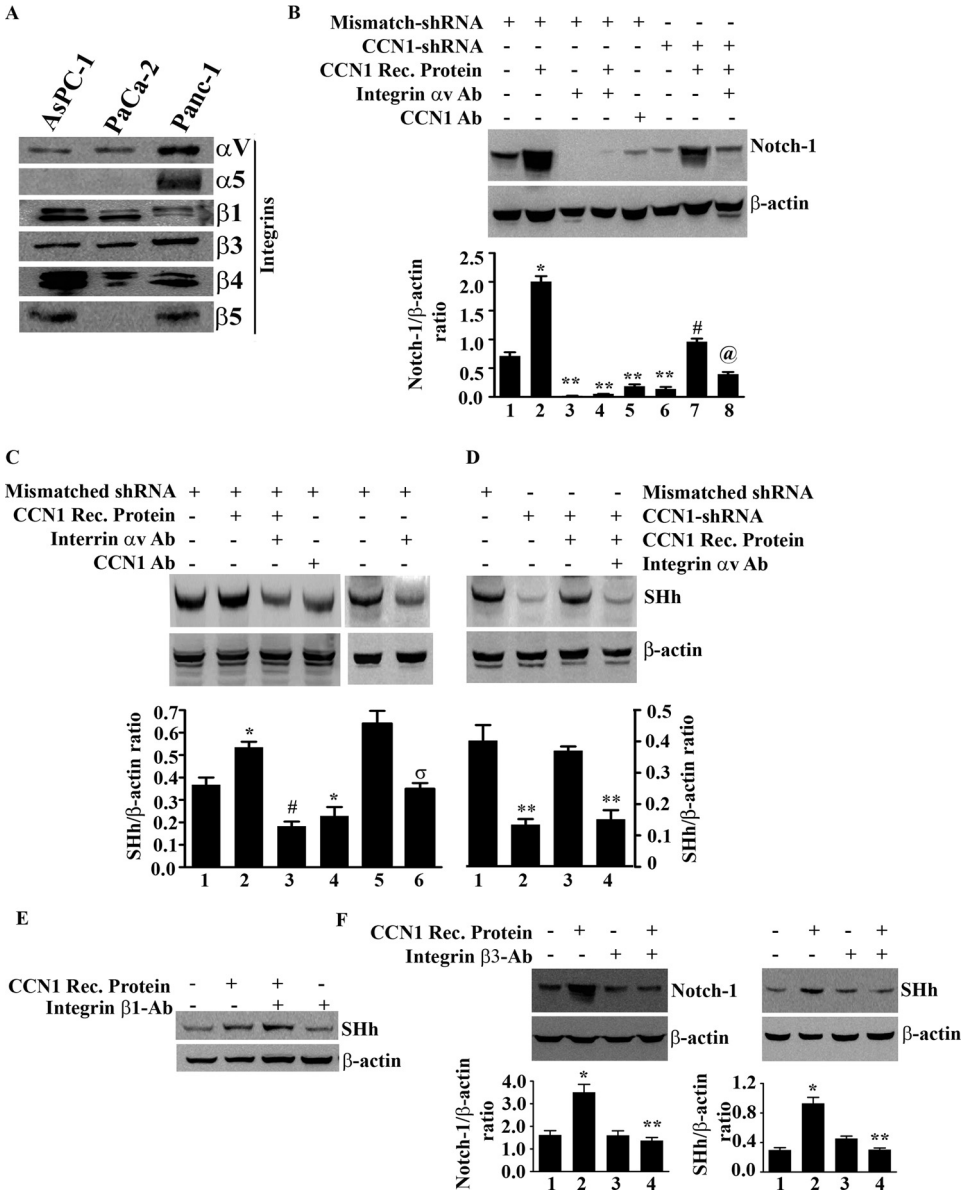

FIGURE 6. **CCN1-induced *Notch-1* activation and SHh overexpression are mediated through integrin $\alpha v\beta 3$ receptor in Panc-1.** *A*, representative Western blots show the expression of different subunits of integrins (α and β) in different pancreatic cell lines. The cell lysates of indicated pancreatic cancer cell lines were Western blotted with different subunits of α and β integrins. *B*, representative Western blots illustrate the expression of *Notch-1* in Panc-1 cells under different treatment conditions (*i.e.* mismatched control, CCN1-silenced, CCN1 recombinant protein-treated, CCN1-silenced cells treated with CCN1 recombinant protein, integrin αv antibody treated, integrin αv antibody, and CCN1 recombinant protein treated, CCN1-silenced cells treated with integrin αv antibody, and CCN1 recombinant protein and CCN1 antibody alone). The bar diagram represents the relative expression of Notch1 in the same samples. Error bars indicate ± S.D. of three independent experiments. *, $p < 0.001$ *versus* mismatched control (*mc*), **, $p < 0.00024$ *versus* mc, #, $p < 0.032$ *versus lane 1* and @, $p < 0.01$ *versus lane 7*. *C* and *D*, representative Western blots illustrate the expression of SHh in Panc-1 cells under different treatment conditions as indicated above. The bar graph represents the relative expression of SHh in different experimental conditions. Error bars indicate ± S.D. of three independent experiments. *, $p < 0.05$ *versus* mc, #, $p < 0.0054$ *versus lane 2*, σ, $p < 0.0064$ *versus lane 5* (mc), and **, $p < 0.001$ *versus lane 1* (mc). *E*, representative immunoblots show the effect of integrin $\beta 1$ antibody on SHh in Panc-1 cells under different treatment conditions. Note, $\beta 1$ shows no effect on CCN1-induced SHh expression. *F*, representative immunoblots show the effect of integrin $\beta 3$ antibody on *Notch-1* and SHh in Panc-1 cells under different treatment conditions. Note, $\beta 3$ blocks CCN1-induced SHh expression. *, $p < 0.01$ *versus lane 1* and **, $p < 0.02$ *versus lane 2*.

motility of MIA-PaCa-2^{CCN1KO} cells in the presence or absence of CCN1 recombinant protein with or without cyclopamine (an inhibitor of SHh signaling). As expected, the addition of CCN1 recombinant protein in the culture media helped in recovering the motile behavior of MIA-PaCa-2^{CCN1KO} cells parallel with the induction of SHh expression, which was noticeably absent due to the shRNA-mediated silencing of CCN1 (Fig. 7*D*, *upper panel*). The increased motility of MIA-PaCa-2^{CCN1KO} cells by recombinant CCN1 protein can be abrogated by concomitant treatment with cyclopamine without altering the expression of SHh (Fig. 7*D*, *lower panel*). Collectively, these findings suggest that CCN1 induced motility of pancreatic cancer cells is SHh signaling dependent.

DISCUSSION

The importance of SHh signaling in the development of pancreatic cancer and chemoresistance has fueled intensive study on this signaling molecule (48). Ample evidence indicates that

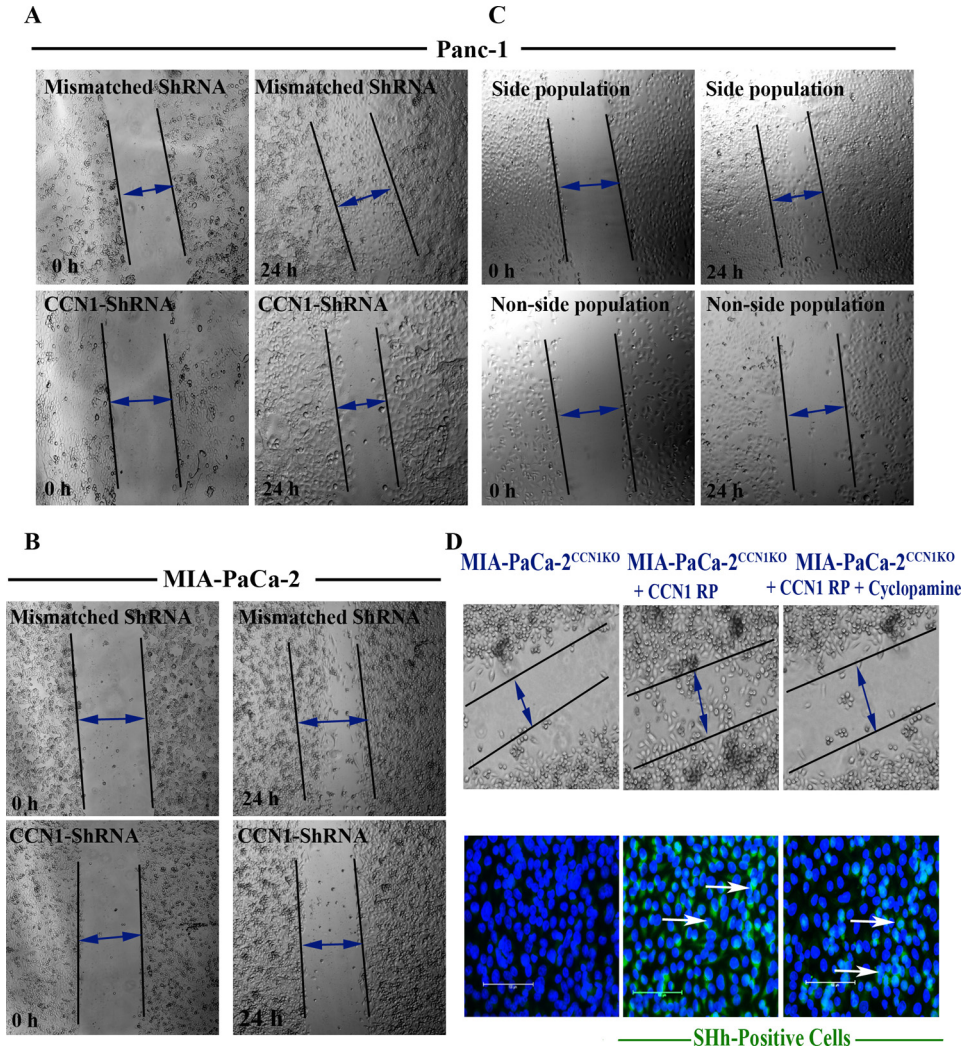

FIGURE 7. **Evaluation of cellular motility (Scratch wound assay) in different pancreatic cell lines under the CCN1 microenvironment.** *A* and *B*, phase-contrast microphotographs represent the motility pattern in mismatched and CCN1 knock-out Panc-1 and MIA-PaCa-2 cells at 0 and 24 h, respectively. *C*, phase-contrast microphotographs exhibit the motility pattern in side population and non-side population of Panc-1 cells at 0 and 24 h, respectively. *D*, phase-contrast microphotographs represent the motility pattern of CCN1 knock-out Mia-PaCa-2 cells, CCN1 recombinant protein (100 ng/ml)-treated CCN1 knock-out MIA-PaCa-2 cells and CCN1 recombinant protein and Smo inhibitor, Cyclopamine (5.0 μM)-treated CCN1 knock-out MIA-PaCa-2 cells, respectively. Microphotographs (*lower panel*) show the immunoexpression of SHh in the above treated samples using immunofluorescence.

SHh signaling is one of the "underpinning" signaling pathways which is aberrantly overexpressed in virtually all PDAC and is associated with tumor growth, metastasis and less survival in a genetically engineered mouse model of pancreatic cancer and an orthotropic xenograft model. Additionally, the viability of pancreatic cancer stem cells is also dependant on sustained expression of SHh signaling. Thus, blockading the SHh signaling pathway with an inhibitor reduces pancreatic cancer growth and improves outcomes (19, 48). Recently, we found that CCN1 functions in a similar pathobiological role in pancreatic carcinogenesis, indicating that CCN1 signaling is critical for epithelial-mesenchymal transition and stemness and is required to promote tumor growth by the side population (cancer stem cells) of pancreatic cancer cells in a xenograft model (7). In our present work, we sought to unravel whether and how these two signaling complexes interact or associate to orchestrate the dynamic events linked with the development of pancreatic cancer. Our studies reveal that CCN1 is an upstream regulator of SHh, modulating SHh expression through the integrin $\alpha v\beta 3$-*Notch-1* signaling pathway (Fig. 8).

These studies have highlighted that the expression levels of CCN1 and SHh are markedly higher in aggressive pancreatic cell lines (*i.e.* Panc-1, Hs766T, and MIA-PaCa-2) as compared with less aggressive cells (Fig. 1). The immunohistochemical studies established that the expressions of CCN1 and SHh were first detected in the PanIN1 stage, a precursor lesion (8), and both proteins are persistently expressed at higher levels as lesions progress to more advanced stages. Furthermore, these studies show that CCN1 tightly controls SHh signaling in pancreatic cancer cells. We demonstrated that silencing of CCN1 inhibits the expressions of SHh and its downstream signaling proteins and effectors in pancreatic cancer cells, and it simultaneously blocks the *in vitro* migration of these cells, while SHh inhibition exhibits no effect on CCN1 expression but minimizes the migration of pancreatic cancer cells (Figs. 2 and 3). Collectively, these studies suggest that SHh lies downstream of

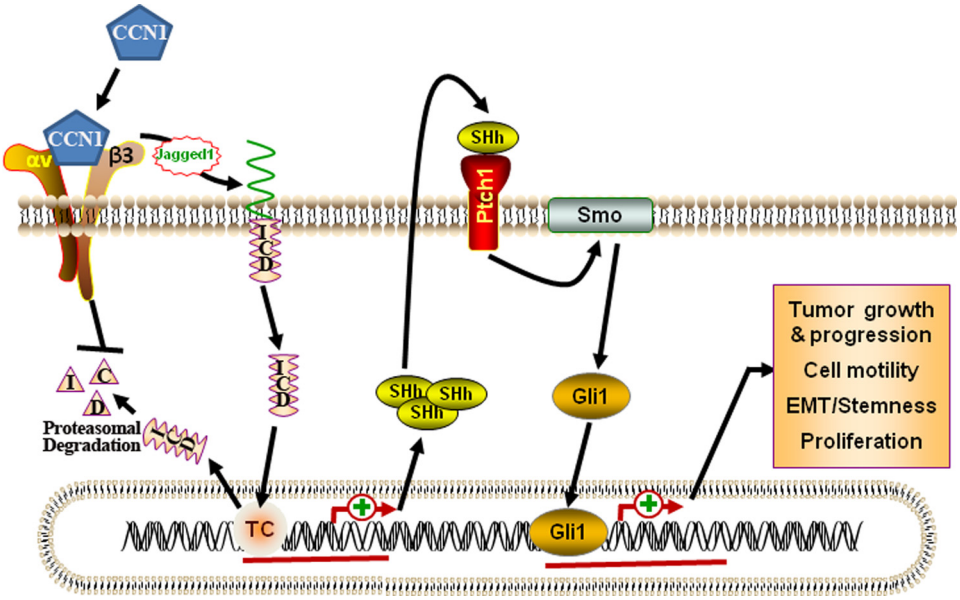

FIGURE 8. **The diagram illustrates the possible mechanism of tumor cells-secreted CCN1 in regulation of pancreatic carcinogenesis through the regulation of SHh signaling.** The studies postulated that CCN1-mediated induction of SHh is regulated via integrin-$\alpha v\beta 3$-*Notch-1*-signaling pathway. CCN1 recruits active *Notch-1* for the regulation of SHh by stabilizing the active *Notch-1* receptor through blocking the proteasomal degradation process. The studies also speculate that Jagged1, one of the ligands of *Notch-1*, may participate in CCN1-induced activation of *Notch-1* as Jagged1 is regulated by CCN1 in pancreatic cancer cells (supplemental Fig. S2). *ICD*: notch-1 intracellular domain, and *TC*: transcriptional complex.

CCN1 and therefore, CCN1 can be considered a required signaling molecule for the initiation and progression of pancreatic carcinogenesis and drug resistance since SHh acts at multiple stages during pancreatic carcinogenesis and increases drug resistance (10, 11, 14, 38).

Different laboratories including ours have characterized the side population (SP) of pancreatic cancer cells (7, 49–51). Like other tumor cell lines, the SP of pancreatic cancer cell lines was enriched with cancer stem cells, exhibited an elevated migratory feature (Fig. 3) and formed tumors in the xenograft model (7). Given the pathobiological importance of the SP, we compared the tumorigenic potency of the SP and NSP in the mouse xenograft and determined the status of CCN1 and SHh in these xenografts. As expected, the SP cells formed larger tumors more quickly as compared with NSP when injected 1×10^6 cells/mouse for 45 days. Further, the SP xenograft highly expressed CCN1 and SHh and downstream signaling partners as compared with the NSP xenograft (Fig. 3). Collectively, these studies strengthen the above perception and demonstrate a plausible connection between CCN1 and SHh signaling.

Given the activation of SHh by CCN1 in pancreatic cancer cells, how does CCN1 regulate SHh to orchestrate pathobiological functions such as cellular motility/migration? We envision at least one potential scenario: the participation of active *Notch*-1, a regulator of pancreatic tumorigenesis (40), as SHh is activated by *Notch-1* or participates in *Notch-1* signaling pathway in different biological contexts (41, 52) and CCN1 activates the *Notch-1* in pancreatic cancer cells (Fig. 5) (7). Additionally, without interacting with CCN1, the "*Notch* activation process" inhibitor DAPT (γ-secretase inhibitor) blocks SHh expression in pancreatic cancer cells (Fig. 4), suggesting a unique signaling map in pancreatic carcinogenesis in which CCN1 regulates SHh through active *Notch-1*. However, it is still uncertain how CCN1 activates *Notch-1* in this scenario. It could be mediated by transcriptional or posttranscriptional/posttranslational, or even both mechanisms. The transcriptional regulation can be ruled out because our preliminary qPCR analysis indicates that the *Notch-1* mRNA expression is not significantly decreased in CCN1 lacking Panc-1 cells as compared with CCN1 expressing Panc-1 cells (data not included). Thus, it could be mediated by a post-transcriptional/post-translational mechanism. The post-transcriptional/post-translational regulation of *Notch-1* is a complex multi-step process. These include maturation, activation, and finally proteasomal degradation after the transcriptional regulation of target genes (43–45, 53, 55, 56). Our studies indicate that CCN1 inhibits the proteasomal degradation process to keep *Notch-1*(ICD) stable and active in pancreatic cancer cells (Fig. 5). *Notch-1* activation, which is a process of releasing an intracellular domain of Notch (ICD) from the membrane into the cytoplasm, is primarily mediated by complex interactions of receptor Notch and ligands (*i.e.* DLL4, Jagged1 and Jagged2) at the surface of adjacent cells accompanied by a proteolytic process (54). Based on the preliminary results, which demonstrated shRNA-based CCN1 silencing inhibits Jagged1 expression in pancreatic cancer cells (supplemental Fig. S2), we cannot rule out the possibility that CCN1-induced activation of *Notch*-1 could be mediated through the induction of Jagged1 expression in these cells. The hypothesis is now under investigation in our laboratory.

CCN1 promotes cell adhesion, migration, proliferation, apoptosis, and angiogenesis under specific environmental conditions. It exerts its functions primarily through direct binding to integrin receptors with different combinations. For example, CCN1 promotes cell survival through integrin $\alpha v\beta 3$, but apoptosis is induced through $\alpha 6\beta 1$ (24). These reports persuaded us to investigate whether CCN1, to promote an invasive phenotype such as cell motility (Fig. 7), modulates *Notch-1* followed by SHh through the direct binding with distinct integrin

receptors. We found that integrin $\alpha v\beta 3$is sufficient to regulate *Notch-1* and SHh by CCN1 in pancreatic cancer cells (Fig. 6). We are currently investigating the role of other possible integrin subunits, if any, in pancreatic carcinogenesis.

In summary, we have identified CCN1 as a key regulator for SHh signaling in pancreatic carcinogenesis and the studies have resolved how the CCN1 system becomes rewired at the molecular and cellular levels to promote pancreatic cancer. Moreover, our current studies not only succeeded in helping to illuminate the role of CCN1 in pancreatic cancer development, but also, they should facilitate the harnessing of CCN1 to treat pancreatic cancer.

Acknowledgments—We thank all the members of CRU for help in generating this manuscript.

REFERENCES

1. Hruban, R. H., Iacobuzio-Donahue, C., Wilentz, R. E., Goggins, M., and Kern, S. E. (2001) Molecular pathology of pancreatic cancer. *Cancer J.* **7,** 251–258
2. McCarthy, D. M., Brat, D. J., Wilentz, R. E., Yeo, C. J., Cameron, J. L., Kern, S. E., and Hruban, R. H. (2001) Pancreatic intraepithelial neoplasia and infiltrating adenocarcinoma: analysis of progression and recurrence by DPC4 immunohistochemical labeling. *Hum. Pathol.* **32,** 638–642
3. Wilentz, R. E., Saavedra, J. A., and Hruban, R. H. (2001) in *Exocrine Pancreas, Pathology of Incipient Neoplasia* (Henson, D. E., and Saavedra, J. A., eds), Oxford Press, New York
4. Kern, S. E., Shi, C., and Hruban, R. H. (2011) The complexity of pancreatic ductal cancers and multidimensional strategies for therapeutic targeting. *J. Pathol.* **223,** 295–306
5. Maitra, A., Kern, S. E., and Hruban, R. H. (2006) Molecular pathogenesis of pancreatic cancer. *Best. Pract. Res. Clin. Gastroenterol.* **20,** 211–226
6. Hruban, R. H., Goggins, M., Parsons, J., and Kern, S. E. (2000) Progression model for pancreatic cancer. *Clin. Cancer Res.* **6,** 2969–2972
7. Haque, I., Mehta, S., Majumder, M., Dhar, K., De, A., McGregor, D., Van Veldhuizen, P. J., Banerjee, S. K., and Banerjee, S. (2011) Cyr61/CCN1 signaling is critical for epithelial-mesenchymal transition and stemness and promotes pancreatic carcinogenesis. *Mol. Cancer* **10,** 8
8. Berman, D. M., Karhadkar, S. S., Maitra, A., Montes De Oca, R., Gerstenblith, M. R., Briggs, K., Parker, A. R., Shimada, Y., Eshleman, J. R., Watkins, D. N., and Beachy, P. A. (2003) Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature* **425,** 846–851
9. Beachy, P. A., Karhadkar, S. S., and Berman, D. M. (2004) Mending and malignancy. *Nature* **431,** 402
10. Morton, J. P., Mongeau, M. E., Klimstra, D. S., Morris, J. P., Lee, Y. C., Kawaguchi, Y., Wright, C. V., Hebrok, M., and Lewis, B. C. (2007) Sonic hedgehog acts at multiple stages during pancreatic tumorigenesis. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 5103–5108
11. Hidalgo, M., and Maitra, A. (2009) The hedgehog pathway and pancreatic cancer. *N. Engl. J. Med.* **361,** 2094–2096
12. Thayer, S. P., di Magliano, M. P., Heiser, P. W., Nielsen, C. M., Roberts, D. J., Lauwers, G. Y., Qi, Y. P., Gysin, S., Fernández-del Castillo, C., Yajnik, V., Antoniu, B., McMahon, M., Warshaw, A. L., and Hebrok, M. (2003) Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* **425,** 851–856
13. Kim, S. K., and Hebrok, M. (2001) Intercellular signals regulating pancreas development and function. *Genes Dev.* **15,** 111–127
14. Morton, J. P., and Lewis, B. C. (2007) Shh signaling and pancreatic cancer: implications for therapy? *Cell Cycle* **6,** 1553–1557
15. Dimou, A., Syrigos, K., and Saif, M. W. (2011) Rationale for inhibition of the hedgehog pathway paracrine loop in pancreatic adenocarcinoma. *JOP.* **12,** 1–5
16. Harris, L. G., Samant, R. S., and Shevde, L. A. (2011) Hedgehog signaling: networking to nurture a promalignant tumor microenvironment. *Mol. Cancer Res.* **9,** 1165–1174
17. Villavicencio, E. H., Walterhouse, D. O., and Iannaccone, P. M. (2000) The sonic hedgehog-patched-gli pathway in human development and disease. *Am. J. Hum. Genet.* **67,** 1047–1054
18. Feldmann, G., Dhara, S., Fendrich, V., Bedja, D., Beaty, R., Mullendore, M., Karikari, C., Alvarez, H., Iacobuzio-Donahue, C., Jimeno, A., Gabrielson, K. L., Matsui, W., and Maitra, A. (2007) Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers. *Cancer Res.* **67,** 2187–2196
19. Gupta, S., Takebe, N., and Lorusso, P. (2010) Targeting the Hedgehog pathway in cancer. *Ther. Adv. Med. Oncol.* **2,** 237–250
20. Olive, K. P., Jacobetz, M. A., Davidson, C. J., Gopinathan, A., McIntyre, D., Honess, D., Madhu, B., Goldgraben, M. A., Caldwell, M. E., Allard, D., Frese, K. K., Denicola, G., Feig, C., Combs, C., Winter, S. P., Ireland-Zecchini, H., Reichelt, S., Howat, W. J., Chang, A., Dhara, M., Wang, L., Rückert, F., Grützmann, R., Pilarsky, C., Izeradjene, K., Hingorani, S. R., Huang, P., Davies, S. E., Plunkett, W., Egorin, M., Hruban, R. H., Whitebread, N., McGovern, K., Adams, J., Iacobuzio-Donahue, C., Griffiths, J., and Tuveson, D. A. (2009) Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* **324,** 1457–1461
21. Bailey, J. M., Swanson, B. J., Hamada, T., Eggers, J. P., Singh, P. K., Caffery, T., Ouellette, M. M., and Hollingsworth, M. A. (2008) Sonic hedgehog promotes desmoplasia in pancreatic cancer. *Clin. Cancer Res.* **14,** 5995–6004
22. Brigstock, D. R. (2003) The CCN family: a new stimulus package. *J. Endocrinol.* **178,** 169–175
23. Perbal, B. (2004) CCN proteins: multifunctional signaling regulators. *Lancet* **363,** 62–64
24. Jun, J. I., and Lau, L. F. (2011) Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. *Nat. Rev. Drug Discov.* **10,** 945–963
25. Lau, L. F. (2011) CCN1/CYR61: the very model of a modern matricellular protein. *Cell Mol. Life Sci.* **68,** 3149–3163
26. Banerjee, S.K., and Banerjee, S. (2012) CCN5/WISP-2: A micromanager of breast cancer progression. *J. Cell Commun. Signal.* **6,** 63–71
27. Kireeva, M. L., MO, F. E., Yang, G. P., and Lau, L. F. (1996) Cyr61, a product of a growth factor-inducible immediate-early gene, promotes cell proliferation, migration, and adhesion. *Mol. Cell. Biol.* **16,** 1326–1334
28. Tong, X., O'Kelly, J., Xie, D., Mori, A., Lemp, N., McKenna, R., Miller, C. W., and Koeffler, H. P. (2004) Cyr61 suppresses the growth of non-small-cell lung cancer cells via the beta-catenin-c-myc-p53 pathway. *Oncogene* **23,** 4847–4855
29. Sampath, D., Zhu, Y., Winneker, R. C., and Zhang, Z. (2001) Aberrant expression of Cyr61, a member of the CCN (CTGF/Cyr61/Cef10/NOVH) family, and dysregulation by 17 beta-estradiol and basic fibroblast growth factor in human uterine leiomyomas. *J. Clin. Endocrinol. Metab.* **86,** 1707–1715
30. Tsai, M. S., Bogart, D. F., Li, P., Mehmi, I., and Lupu, R. (2002) Expression and regulation of Cyr61 in human breast cancer cell lines. *Oncogene* **21,** 964–973
31. Xie, D., Yin, D., Tong, X., O'Kelly, J., Mori, A., Miller, C., Black, K., Gui, D., Said, J. W., and Koeffler, H. P. (2004) Cyr61 is overexpressed in gliomas and involved in integrin-linked kinase-mediated Akt and β-catenin-TCF/Lef signaling pathways. *Cancer Res.* **64,** 1987–1996
32. Ray, G., Banerjee, S., Saxena, N. K., Campbell, D. R., Van, Veldhuizen, P., and Banerjee, S. K. (2005) Stimulation of MCF-7 tumor progression in athymic nude mice by 17β-estradiol induces WISP-2/CCN5 expression in xenografts: a novel signaling molecule in hormonal carcinogenesis. *Oncol. Rep.* **13,** 445–448
33. Kambhampati, S., Banerjee, S., Dhar, K., Mehta, S., Haque, I., Dhar, G., Majumder, M., Ray, G., Vanveldhuizen, P. J., and Banerjee, S. K. (2010) 2-methoxyestradiol inhibits Barrett's esophageal adenocarcinoma growth and differentiation through differential regulation of the β-catenin-E-cadherin axis. *Mol. Cancer Ther.* **9,** 523–534
34. Banerjee, S., Sengupta, K., Dhar, K., Mehta, S., D'Amore, P. A., Dhar, G., and Banerjee, S. K. (2006) Breast cancer cells secreted platelet-derived

growth factor-induced motility of vascular smooth muscle cells is mediated through neuropilin-1. *Mol. Carcinog.* **45,** 871–880
35. Banerjee, S., Dhar, G., Haque, I., Kambhampati, S., Mehta, S., Sengupta, K., Tawfik, O., Phillips, T. A., and Banerjee, S. K. (2008) CCN5/WISP-2 expression in breast adenocarcinoma is associated with less frequent progression of the disease and suppresses the invasive phenotypes of tumor cells. *Cancer Res.* **68,** 7606–7612
36. Sengupta, K., Banerjee, S., Saxena, N. K., and Banerjee, S. K. (2004) Thombospondin-1 disrupts estrogen-induced endothelial cell proliferation and migration and its expression is suppressed by estradiol. *Mol. Cancer Res.* **2,** 150–158
37. Dhar, G., Mehta, S., Banerjee, S., Gardner, A., McCarty, B. M., Mathur, S. C., Campbell, D. R., Kambhampati, S., and Banerjee, S. K. (2007) Loss of WISP-2/CCN5 signaling in human pancreatic cancer: a potential mechanism for epithelial-mesenchymal-transition. *Cancer Lett.* **254,** 63–70
38. Yao, J., An, Y., Wie, J. S., Ji, Z. L., Lu, Z. P., Wu, J. L., Jiang, K. R., Chen, P., Xu, Z. K., and Miao, Y. (2011) Cyclopamine reverts acquired chemoresistance and down-regulates cancer stem cell markers in pancreatic cancer cell lines. *Swiss. Med. Wkly.* **141,** w13208
39. Harris, L. G., Pannell, L. K., Singh, S., Samant, R. S., and Shevde, L. A. (2012) Increased vascularity and spontaneous metastasis of breast cancer by hedgehog signaling mediated upregulation of cyr61. *Oncogene* **31,** 3370–3380
40. Bolós, V., Grego-Bessa, J., and de la Pompa, J. L. (2007) Notch signaling in development and cancer. *Endocr. Rev.* **28,** 339–363
41. Morrow, D., Cullen, J. P., Liu, W., Guha, S., Sweeney, C., Birney, Y. A., Collins, N., Walls, D., Redmond, E. M., and Cahill, P. A. (2009) Sonic Hedgehog induces Notch target gene expression in vascular smooth muscle cells via VEGF-A. *Arterioscler. Thromb. Vasc. Biol.* **29,** 1112–1118
42. Leong, K. G., Niessen, K., Kulic, I., Raouf, A., Eaves, C., Pollet, I., and Karsan, A. (2007) Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin. *J. Exp. Med.* **204,** 2935–2948
43. Gupta-Rossi, N., Le Bail, O., Gonen, H., Brou, C., Logeat, F., Six, E., Ciechanover, A., and Israël, A. (2001) Functional interaction between SEL-10, an F-box protein, and the nuclear form of activated Notch1 receptor. *J. Biol. Chem.* **276,** 34371–34378
44. Lai, E. C. (2002) Protein degradation: four E3s for the notch pathway. *Curr. Biol.* **12,** R74–R78
45. Mo, J. S., Kim, M. Y., Han, S. O., Kim, I. S., Ann, E. J., Lee, K. S., Seo, M. S., Kim, J. Y., Lee, S. C., Park, J. W., Choi, E. J., Seong, J. Y., Joe, C. O., Faessler, R., and Park, H. S. (2007) Integrin-linked kinase controls Notch1 signaling by down-regulation of protein stability through Fbw7 ubiquitin ligase. *Mol. Cell. Biol.* **27,** 5565–5574
46. Yoo, Y. A., Kang, M. H., Kim, J. S., and Oh, S. C. (2008) Sonic hedgehog signaling promotes motility and invasiveness of gastric cancer cells through TGF-β-mediated activation of the ALK5-Smad 3 pathway. *Carcinogenesis* **29,** 480–490
47. Bailey, J. M., Mohr, A. M., and Hollingsworth, M. A. (2009) Sonic hedgehog paracrine signaling regulates metastasis and lymphangiogenesis in pancreatic cancer. *Oncogene* **28,** 3513–3525
48. Feldmann, G., Rauenzahn, S., and Maitra, A. (2009) *In vitro* models of pancreatic cancer for translational oncology research. *Expert. Opin. Drug Discov.* **4,** 429–443
49. Wang, Y. H., Li, F., Luo, B., Wang, X. H., Sun, H. C., Liu, S., Cui, Y. Q., and Xu, X. X. (2009) A side population of cells from a human pancreatic carcinoma cell line harbors cancer stem cell characteristics. *Neoplasma* **56,** 371–378
50. Kabashima, A., Higuchi, H., Takaishi, H., Matsuzaki, Y., Suzuki, S., Izumiya, M., Iizuka, H., Sakai, G., Hozawa, S., Azuma, T., and Hibi, T. (2009) Side population of pancreatic cancer cells predominates in TGF-β-mediated epithelial to mesenchymal transition and invasion. *Int. J. Cancer* **124,** 2771–2779
51. Zhou, J., Wang, C. Y., Liu, T., Wu, B., Zhou, F., Xiong, J. X., Wu, H. S., Tao, J., Zhao, G., Yang, M., and Gou, S. M. (2008) Persistence of side population cells with high drug efflux capacity in pancreatic cancer. *World J. Gastroenterol.* **14,** 925–930
52. López, S. L., Paganelli, A. R., Siri, M. V., Ocaña, O. H., Franco, P. G., and Carrasco, A. E. (2003) Notch activates sonic hedgehog and both are involved in the specification of dorsal midline cell-fates in *Xenopus*. *Development* **130,** 2225–2238
53. Mumm, J. S., and Kopan, R. (2000) Notch signaling: from the outside in. *Dev. Biol.* **228,** 151–165
54. Li, J. L., and Harris, A. L. (2005) Notch signaling from tumor cells: a new mechanism of angiogenesis. *Cancer Cell* **8,** 1–3
55. Kim, M. Y., Mo, J. S., Ann, E. J., Yoon, J. H., Jung, J., Choi, Y. H., Kim, S. M., Kim, H. Y., Ahn, J. S., Kim, H., Kim, K., Hoe, H. S., and Park, H. S. (2011) Regulation of Notch1 signaling by the APP intracellular domain facilitates degradation of the Notch1 intracellular domain and RBP-Jk. *J. Cell Sci.* **124,** 1831–1843
56. Kim, M. Y., Mo, J. S., Ann, E. J., Yoon, J. H., and Park, H. S. (2012) Dual regulation of notch1 signaling pathway by adaptor protein fe65. *J. Biol. Chem.* **287,** 4690–4701